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Living in isolation for almost 40 years: molecular divergence of the 28S rDNA and COI sequences between French and Polish populations of the cave beetle *Speonomus normandi hydrophilus* (Jeannel, 1907)

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Abstract

The paper gives the results of the first studies on the molecular divergence between native and non-native populations of *Speonomus normandi hydrophilus* (Jeannel, 1907). This species is endemic to Massif Arize in the Central Pyrenees (France), and represents highly specialised organisms that live underground. In 1982, one hundred specimens of *S. normandi hydrophilus* had been experimentally introduced into the Dzwonnica Cave (Poland). Since then, a numerous population has developed in the Towarna-Dzwonnica cave system, and the neighbouring Cabanowa Cave. After almost 40 years of isolation between native and non-native populations, the genetic variations were examined using the COI and 28S rDNA genes. Analyses of the haplotypes of 28S showed one common haplogroup, which confirms the origin of the Polish group. The differentiation of haplotypes for the COI marker was high for both the French and Polish populations. Altogether 18 haplotypes of this marker have been detected, 12 in the French population and 9 in the Polish. However, only a portion of the haplotypes is shared between the native and introduced population.

Keywords

Coleoptera, intentionally introduced species, mitochondrial DNA, nuclear DNA, population genetics, troglobites

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Introduction

Recently, due to human activities, such as high-speed transport systems and increased shipping, insect species are moving across ecosystems, countries, and continents (e.g., Gogala 2003; Harmat et al. 2006; Lis et al. 2008; Lis and Whitehead 2019). Most species are introduced accidentally to new areas, including the Asian tiger mosquito *Aedes albopictus* (Skuse, 1895), spotted lanternfly *Lycorma delicatula* (White, 1845), and brown marmorated stink bug *Halyomorpha halys* (Stål, 1855). However, some species have been intentionally introduced for various reasons. Several bee species have been introduced as honey producers, i.e., the eastern honey bee *Apis cerana* Fabricius, 1793 from Asia to New Guinea; the honey bee *Apis mellifera* Linnaeus, 1761 from Europe to North America; and the East African lowland honey bee *A. mellifera scutellata* Lepeletier, 1836 from Africa to, for an example, Brazil. Biological pest control is another reason for introducing species into a new environment. For example, the ladybird *Rodolia cardinalis* (Mulsant, 1850) was deliberately transferred from Australia to California to fight the cottony cushion scale *Icerya purchasi* Maskell, 1878, the citrus tree pest (Tomalak and Sosnowska 2008). Another example is the introduction of a butterfly *Cactoblastis cactorum* (Berg, 1885) from Mexico to Australia to combat the prickly pear. The caterpillar of this butterfly species successfully destroyed the unwanted plant (Tomalak and Sosnowska 2008). Species that were accidentally or intentionally introduced to a new environment, created sometimes new populations. They are an important topic in studies of genetic variation, genetic drift, and rapidness of genome changes (Estoup and Clegg 2003; Zepeda-Paulo et al. 2010).

Although the role of isolation in natural populations of cave-dwelling invertebrates has been estimated several times (e.g. Allegrucci et al. 1997; Strecker et al. 2003; Ribera et al. 2010; Kruckenhauser et al. 2011; Bradic et al. 2012; Pérez-Moreno et al. 2017), there exists only a few study on populations intentionally moved from their natural habitat to be implemented in a quite new, non-native cave habitat (Coiffait 1968; Tercafs and Brouwir 1991; Juberthie and Gers 1992; Dethier et al. 2002). Nevertheless, no data on the genetic divergence in intentionally introduced populations isolated for decades have yet been provided.

In the present paper, we provide results on the genetic divergence between native and introduced populations of a cave beetle *Speonomus normandi hydrophilus* (Jeannel 1907), which was intentionally transferred from the French Central Pyrenees to a cave in Poland (Skalski 1994).

S. normandi hydrophilus is a troglobitic species endemic to the Central Pyrenees in France and native populations have been studied by Crouau-Roy (1986, 1990) and Crouau-Roy et al.(1992) in both ecological and genetic aspects. Specimens of the analyzed beetle were brought from France to Poland in 1982 by Skalski and experimentally introduced into the Dzwonnica Cave: the Kraków-Częstochowa Upland, Poland (Skalski 1994). That experiment aimed to study an adaptation of highly specialized species into a new ecosystem, i.e. different biocenosis, climate conditions, and sediments (Skalski 1994).

Materials and methods

Study area

The Bastardech cave "Gouffre de Bastardech" (Ariège) is a small cave with a steep entrance that lies in the Central Pyrenees (France) at an altitude of 630 m a.s.l. (Fig. 1). Its narrow passages are sloping and form two floors, and the walls and floors are covered by sediment (Coiffait 1968).

The Dzwonnica cave, where specimens of *S. normandi hydrophilus* were intentionally introduced, lies in the northern part of the Kraków-Częstochowa Upland, Poland (Fig. 1). As in the native cave, it is also a small cave with a horizontal, narrow passage. It is connected to another cave, the Towarna Cave by a small passage; the latter is situated at 330 m a.s.l (Zygmunt 2013).

Organism

Speonomus normandi hydrophilus (Jeannel 1907) (Coleoptera: Leiodidae), belongs to the tribe Leptodirini, which contains about 900 species (Jeannel 1924; Perreau 2004; Cieslak et al. 2014). This subspecies is endemic to the western part of the Arize Massif in the Central Pyrenees and is a highly specialized organism that lives underground in caves and the mesovoid shallow substratum (Crouau-Roy 1992).

In 1982, Skalski collected 50 males and 50 females of this species from the Bastardech cave in the French Pyrenees. These specimens of both sexes were separately

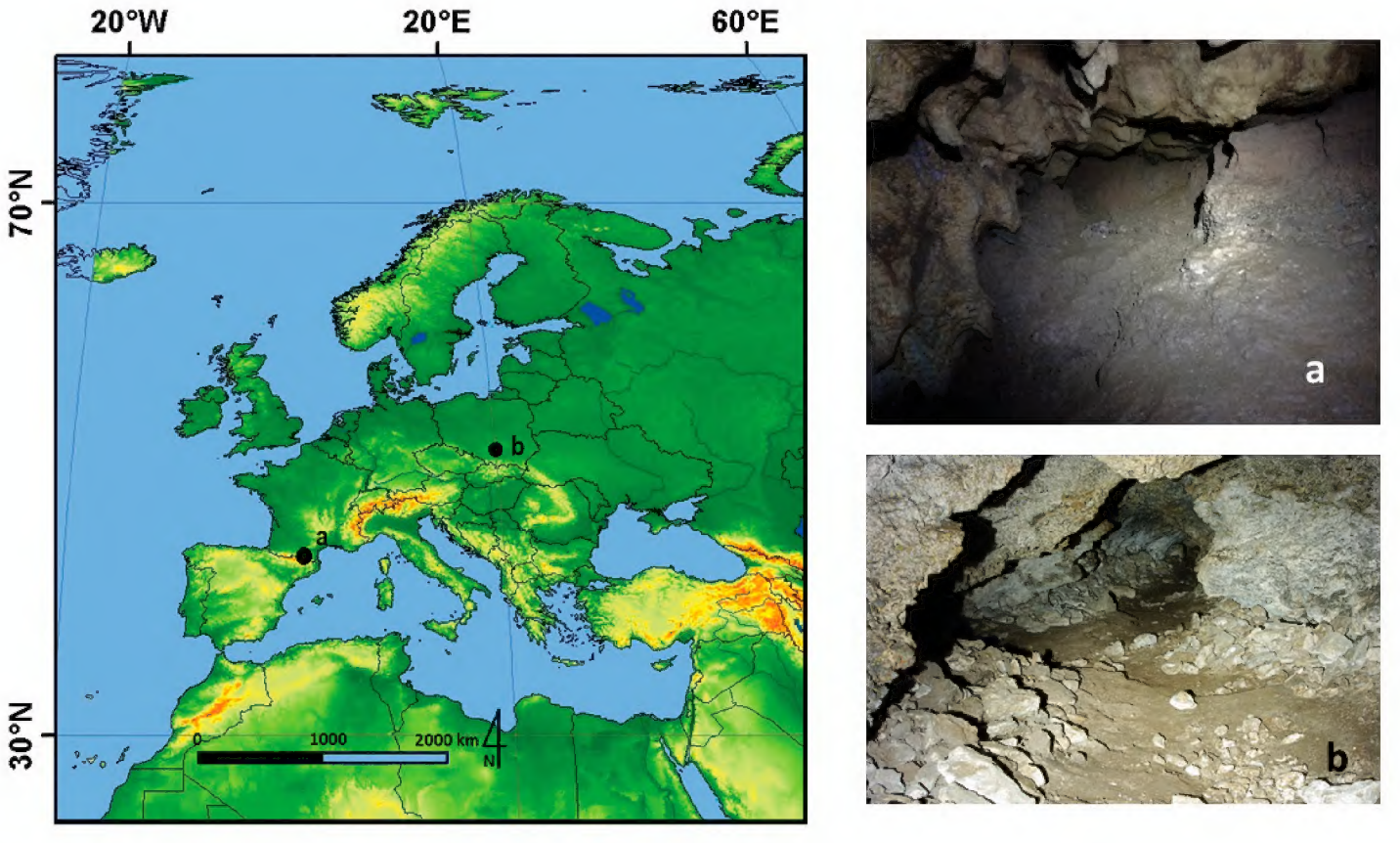

Figure 1. Map of Europe showing the location of sampling sites **a** the Bastardech Cave (France) and its interior **b** the Towarna & Dzwonnica Caves (Poland) and its interior.

inserted into a thermos with ice transported to Poland in two boxes containing cheese. Subsequently, they were introduced to the deep zone of the Dzwonnica cave in Poland. After 12 years, Skalski published that specimens of *S. normandi hydrophilus* had adapted to a new environment and descendants were frequently observed near the place of introduction (Skalski 1994). Nowadays, abundant populations of this species live in three closely situated caves in the Towarne Mts., i.e., the Dzwonnica and the Towarna caves (Klasiński 2006; Dumnicka and Płotek 2013; Kocot-Zalewska 2016) and the Cabanowa cave (unpublished data). The Dzwonnica and Towarna Caves are connected by a passage. The connection between Dzwonnica and Cabanowa is not available for humans but undoubtedly there are rocks crevices penetrable for small organisms.

Specimen sampling

A total of 100 individuals of *S. normandi hydrophilus*, 50 specimens in the Towarna-Dzwonnica caves system (Poland, 49°14'11"N, 19°51'52"E) and 50 specimens in Bastardech Cave (France, 42°57'38"N, 1°14'30"E) were collected by direct searching. In the Bastardech Cave, one week before the sampling, cheese was put onto the cave floor as bait. In the Towarna-Dzwonnica cave system specimens were collected directly without any bait. All samples were stored in 96% ethanol at -20 °C.

Molecular markers

The 549 bp COI fragment was amplified using primer pair Pat and Jerry (Simon et al. 1994). The studied nuclear marker was a 549 bp internal fragment of a large ribosomal unit (LSU), which was amplified using the Ka and Kb primer pair (Ribera et al. 2010). Primers pairs are listed in Table 1.

DNA extraction and amplification

The total genomic DNA was extracted from thorax muscle tissues using a Sherlock AX kit (A&A Biotechnology) following the manufacturer protocol PCR amplifications were performed in a volume of 50 µl using ready-to-use mix PCR Mix Plus (A&A Biotechnology) and primer pairs.

The PCR reaction for the 28S rDNA consisted of initial denaturation for 3 min at 95 °C, followed by 34 cycles of 15 sec at 94 °C, 30 sec at 50 °C, and 40 sec at 72 °C. The final elongation step was 7 min at 72 °C. PCR amplification of the COI consisted

Table 1. Primers used for amplification and sequencing of the 28S and COI genes.

Marker	Primer name	Sequence (5' → 3')	Source
28S	Ka	ACACGGACCAAGGAGTCTAGCATG	Ribera et al. (2010)
	Kb	CGTCCTGCTGTCTTAAGTTAC	Ribera et al. (2010)
COI	Jerry	CAACATTTATTTTGATTTTTTGG	Simon et al. (1994)
	Pat	TCCAATGCACTAATCTGCCATATTA	Simon et al. (1994)

of initial denaturation for 3 min at 94 °C, followed by 34 cycles of 30 sec at 94 °C, 30 sec at 50 °C, 45 sec at 72 °C, and final elongation for 10 min at 72 °C.

The purification of amplicons and sequencing were performed at A&A Biotechnology, Gdynia, Poland. All sequences were obtained in the forward and reverse directions. The obtained sequences were checked using the BLAST tool (https://blast.ncbi.nlm.nih.gov/) to detect possible contamination. All received sequences showed high similarities to sequences of *S. normandi hydrophilus* already deposited into GenBank. The sequences were deposited in GenBank under accession numbers: MW187125–MW187145 and MW187545–MW187565 for the 28S gene and MW187329–MW187352 and MW187448–MW187476 for the COI gene.

Molecular analyses

Each obtained sequence was manually edited for accuracy using FinchTV v. 1.4.0 (Geospiza Inc.) and aligned using ClustalW (with default parameters) in the MEGA X software (Kumar et al. 2018). The ends of sequence reads were trimmed to avoid the influence of missing data resulting from incomplete sequences. The COI sequences were translated to protein sequences to check stop codon in the middle of the sequences and frameshifts. The number of haplotypes (H), haplotype diversity (*hd*), number of segregating sites (S), number of variable sites (V), nucleotide diversity (ND), total number of nucleotide differences (TM), and average number of nucleotide differences (*k*) were computed with DnaSP v.6 (Rozas et al. 2017).The genetic distance between individuals was calculated using a p-distance model in MEGA X software (Kumar et al. 2018).Tajima's D (Tajima 1989) test was performed using DnaSP v.6 (Rozas et al. 2017). The haplotype networks for each gene were constructed using a Median-Joining Network method (Bandelt et. al. 1999) in PopART v.1.7 software (Leigh and Bryant 2015). The visualization of the relationships among haplotypes contains two additional sequences for each gene obtained from GenBank: HG915551.1, AM229403.1 for 28S and HG915401.1, LN849271.1 for COI, respectively.

Results

We successfully obtained 42 sequences (549 bp) of the 28S rDNA and 53 sequences (549 bp) of the COI. In total, 18 haplotypes of the COI were identified among the analyzed specimens. The native Pyrenean population was characterized by 12 different haplotypes, whereas the Polish had nine. Overall, the haplotype diversity of the COI gene (hd) for all specimens was 0.8287 and was 0.8005 for the native French population and 0.8442 for the Polish one.

The average number of nucleotide differences (k) was 5.492, 5.428, and 5.558 for all specimens, the French, and the Polish population, respectively. The total number of the mutations (TM) of all analyzed sequences was 25 and was 21 and 17 for the native Pyrenean population and the Polish one, respectively. All obtained genetic diversity indices of the COI gene are presented in Table 2.

In the case of the nuclear 28S rDNA subunit, three haplotypes were identified, among them, three were detected in the Pyrenean population and a single haplotype within the Polish one. Overall, the haplotype diversity of the 28S rDNA (*hd*) for all analyzed specimens was 0.180. The native French population was characterized by a diversity value of 0.338, while the introduced population had no haplotype diversity. The average number of nucleotide differences (k) was 0.184 for both populations and was 0.352 for the French population. There were no nucleotide differences detected within the Polish population of the 28S rDNA marker. The total number of mutations (TM) was two in the Pyrenean population. No mutations were detected among the population introduced to the Dzwonnica cave in Poland. All obtained genetic diversity indices of the nuclear 28S rDNA marker are presented in Table 3.

The genetic distance between populations for nuclear 28S rDNA and mitochondrial COI markers are presented in Table 4.

The haplotype network generated for 28S gene demonstrate presence of one haplogroup. The most common haplotype (Hap 1) is present in all specimen from Poland, three from France and specimens sequences obtained from GenBank HG915551.1 and AM229403.1 (specimens from Riverenert, not far from Bastardech cave).

Table 2. Genetic diversity indices of the COI gene calculated for the studied specimens.

Population	ns	H	*hd*	S	V	ND	TM	*k*	Tajima's D:
All	53	18	0.8287	25	25	0.01000	25	5.492	-0.00998
France	29	12	0.8005	21	21	0.00989	21	5.428	0.00989
Poland	24	9	0.8442	17	17	0.01012	17	5.558	0.79194

ns – number of analyzed sequences; H- number of haplotypes; *hd* - haplotype diversity, S- number of polymorphic (segregating) sites; V – number of variable sites; ND - nucleotide diversity; TM - total number of mutations; *k* - the average number of nucleotide differences.

Table 3. Genetic diversity indices of the 28S marker calculated for the studied specimens.

Population	ns	H	*hd*	S	V	ND	TM	*k*	Tajima's D:
All	42	3	0.180	2	2	0.00033	2	0.184	-1.12813
France	21	3	0.338	2	2	0.00064	2	0.352	-0.84329
Poland	21	1	0.000	0	0	–	0	0.000	*

ns – number of analyzed sequences; H- number of haplotypes; *hd* - haplotype diversity, S- number of polymorphic (segregating) sites; V – number of variable sites; ND - nucleotide diversity; TM - total number of mutations; *k* - average number of nucleotide differences. Tajima's D statistical significance: Not significant, P > 0.10.*- The DnaSP was unable to conduct Tajima's test.

Table 4. Average pairwise genetic distance, based on the p-distance method, of 28S and COI sequences.

Marker	Population	Overall average pairwise genetic distance
28S	France	0.0009715
	Poland	0.0000000
	All	0.0003343
COI	France	0.0098881
	Poland	0.0101238
	All	0.0100037

The haplotype network analysis for COI gene reveals two distinct haplogroups separated by seven mutational changes. The largest haplogroup (Haplogroup 1) contains 14 different haplotypes and is divided into two subgroups. The subgroup 1 contains the most common haplotype (Hap 2) which is shared among ten specimens from France, seven from Poland and one specimen obtained from GenBank (HG915401.1), from Riverenert. The second subgroup of Haplogroup 1 contains haplotype (Hap 8) which is shared among six specimens from Poland and one from France. The second haplogroup contains the second most common haplotype (Hap 3) which is shared among nine specimens from France and four from Poland. The haplotype Hap19 is referred to the sequence obtained from GenBank LN849271.1, from a cave (Ruisseau souterrain d´Aulot, Saint Girons) located ca 7 km from Bastardech cave in a straight line.

Discussion

The introduction of *S. normandi hydrophilus* to a new environment is not the first experiment of its kind. In the 20th century, many cavernicolous species were experimentally introduced to new caves (Coiffait 1968; Bouillon and Hubart 1982; Tercafs and Brouwir 1991), including three species of the genus *Speonomus* (i.e., *S. diecki*, *S. stygius*, *S. longicornis*). However, this paper gives the first results of genetic variation between the native and introduced populations among *Speonomus* species.

The molecular analyses were carried out using one mitochondrial and one nuclear marker, similar to other studies on genetic variation between closely related species (for example, Niemiller et al. 2008; Faille et al. 2013; Rizzo et al. 2013; Cieslak et al. 2014; Pérez-Moreno et al. 2017) and among one species (for example, Faille et al. 2015; Boyd et al. 2020).

The results obtained in this study showed that nuclear 28S is less diverse than mitochondrial COI, which is unsurprising. Three haplotypes of the 28S marker were detected in the French population but only one in the Polish. Analyses of the haplotype network (Fig. 2) showed one common haplogroup, which confirms the origin of the Polish group.

The variations of haplotypes for the COI marker was high for both the French and Polish populations (0.80 and 0.84, respectively). The most common haplotypes were Hap 2 and Hap 3, which were shared between specimens from both populations (Fig. 3) and formed two major haplogroups.

The high variability in the French population might be explained by the fact that *Speonomus normandi hydrophilus* inhabits caves and the mesovoid shallow substratum in an area of c.a. 30 × 40 km (Crouau-Roy et al. 1992). Many local subpopulations exist, which are not entirely isolated. Thus, some gene flow, although limited, could be observed (Crouau-Roy 1986). Crouau-Roy (1986) findings are reflected in our result of haplotype network for the COI marker (Fig. 3), in which two major groups of haplotypes are visible.

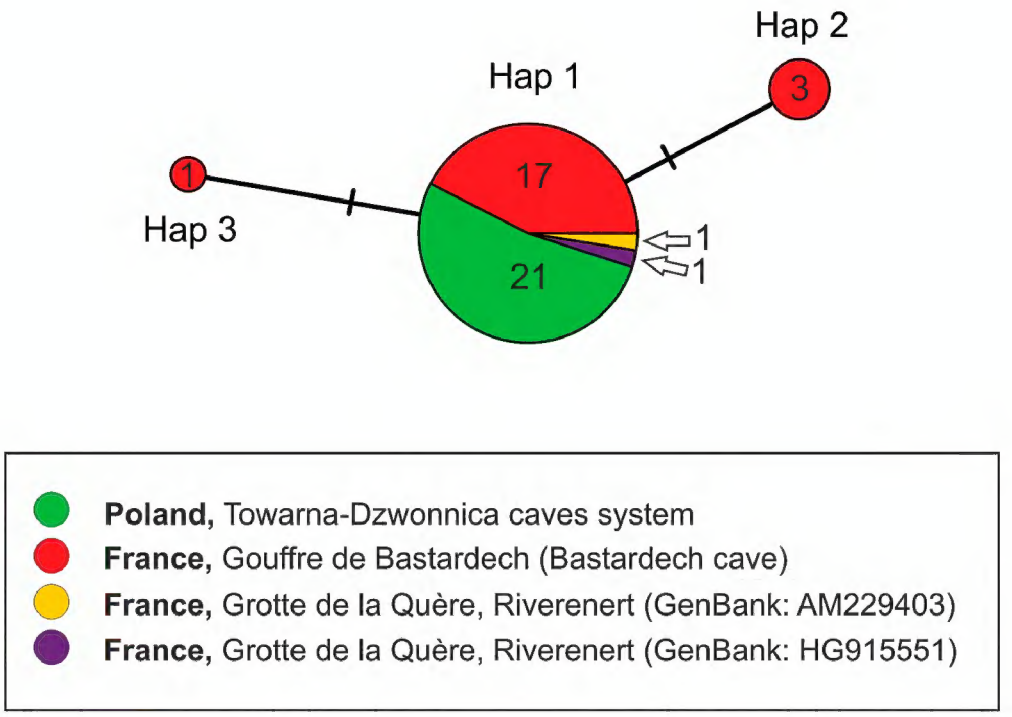

Figure 2. Median-joining haplotype network for 28S sequences. Different colours correspond to geographic origin and circle size is proportional to the number of individuals with the same haplotype. The number of individuals with a specific haplotype is in a circle. Hatch marks along edges represent the number of mutations between nodes.

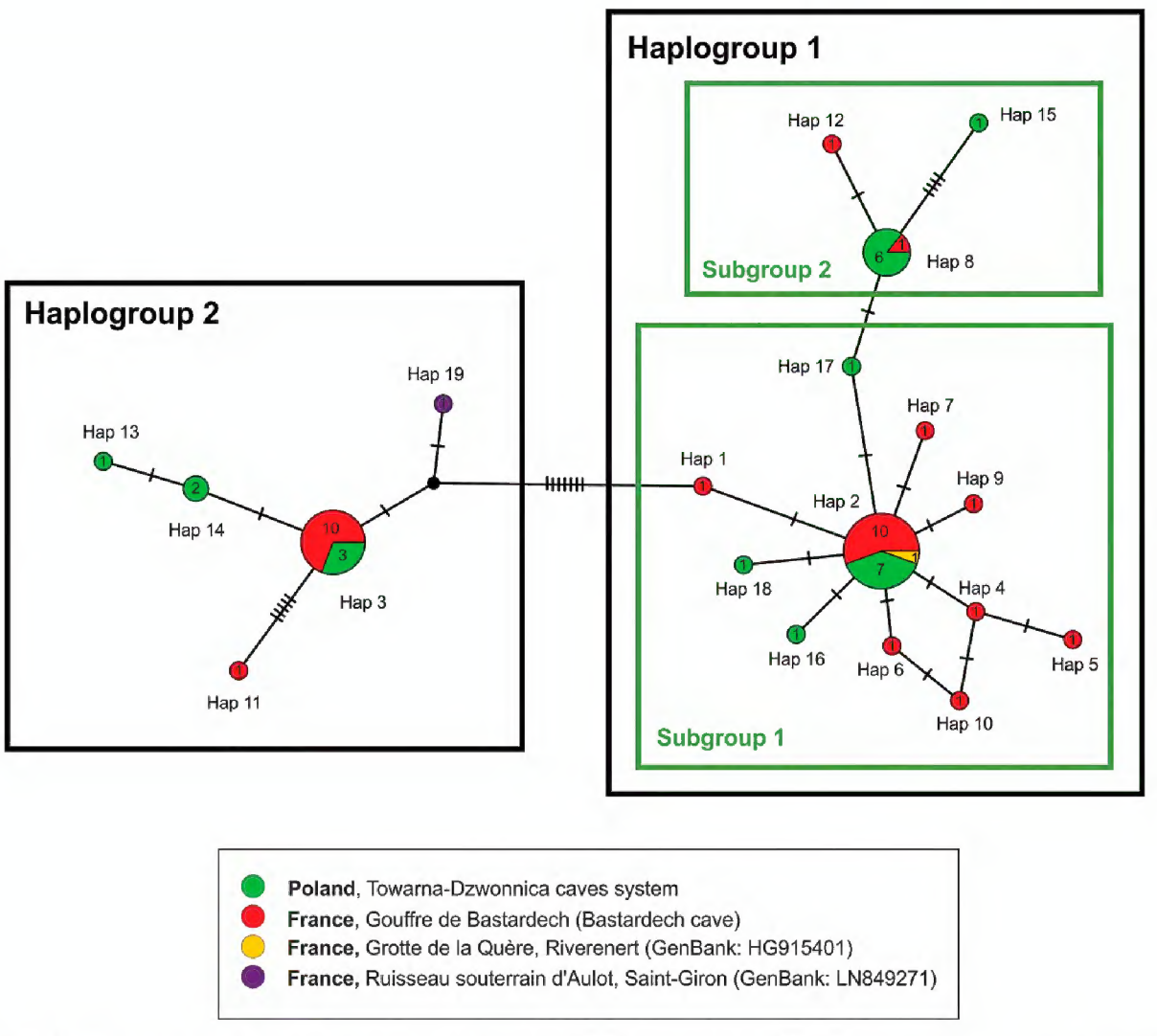

Figure 3. Median-joining haplotype network for COI sequences. Different colours correspond to geographic origin and circle size is proportional to the number of individuals with the same haplotype. The number of individuals with a specific haplotype is in a circle. Hatch marks along edges represent the number of mutations between nodes.

The high diversity of the COI marker among the native population indicates that founder specimens transplanted to Polish cave were also diversified. Currently, the Polish population shares only a portion of the haplotypes with the native French population. Moreover, the presence of specific Polish population haplotypes may be the result of adaptation. Microevolutionary changes in the mtDNA could be stress-induced (Kranthi et al. 2006), and conditions in a new environment can be considered stress factors, which might have been expressed in the mtDNA sequences. For example, air temperature could have had an important influence on current haplotype diversity. The research provided by Crouau-Roy et al. (1992) about populations occurrences in caves and mesovoid shallow substratum (MSS) indicated that caves populations live in a more stable temperature between 7.5 to 12 °C, while MSS populations inhabit areas with a broader temperature spectrum. However, in both population groups, seasonal fluctuations of abundance were observed. Cave specimens were observed throughout the year, whereas the MSS environment were not observed in some months (Crouau-Roy et al. 1992). Stable temperature is present in the Towarna Cave (Kocot-Zalewska 2016). During the research conducted in this cave, 88.3% of the collected specimens inhabited a space with a temperature amplitude 1.8 °C, 11% lived in a place with a temperature amplitude 8.2 °C, and only 0.7% of the collection was caught in place with amplitude 14.6 °C (Kocot-Zalewska 2016). On the other hand, temperature changes may not be the significant survival factor for subterranean organisms. However, the range of tolerated temperatures is limited (Rizzo et al. 2015). Thus, environmental change could lead to the survival of just part of the introduced specimens to the Dzwonnica Cave or it could be a lack of reproduction success among part of the population. Moreover, the observation of the high variability of the COI marker also follows the inheritance of the maternal mitochondrial genome and its fast mutation, which affects the rapidly spreading changes in the native and non-native populations (Kondo et al. 1990; Skibinski et al. 1994; Zhang and Hewitt 2003).

The total number of mutations in the COI was 25, and it was almost equal in both the French and Polish groups (21 ver. 17, respectively). Tajima's D statistic is positive when there is an excess of high-frequency mutations, for example, after a population contraction or under balancing selection. Tajima's D statistic is negative when there is an excess of low-frequency mutations, for instance, after a population expansion, a recent selective sweep, weak negative selection, or when samples come from an admixed population (Stajich and Hahn 2005). In this study, the Tajima's D test was positive for the COI marker results. It had a high value among the Polish group, which might be an effect of high-frequency mutations after a reduction in population (Stajich and Hahn 2005; Jackson et al. 2015). This hypothesis is strongly supported by a small number of specimens who founded a new population (just one hundred individuals) in a new environment. However, this assumption was based only on the COI marker. No polymorphism was found in the 28S rDNA sequences of the Polish population, and therefore, the Tajima test could not be used.

With the observed lower number of haplotypes in the introduced population, the obtained results were not surprising. In the research of Li et al. (2011), five populations

of silver carp (*Hypophthalmichthys molitrix*) were compared between native (China) and introduced (USA, Hungary) habitats. All native populations had a higher variety of haplotypes in comparison to non-natives. Compared to another study on introduced species, a low genetic variation was observed. The amphibian species *Eleutherodactylus johnstonei* (Barbour 1914) was introduced from the Caribbean to Colombia 25 years ago. The recent study, based on two mitochondrial markers, has shown very low genetic variations within the implanted population. There was no variation in 12S rRNA and three haplotypes in the D-loop marker were detected. Interestingly, the two recorded haplotypes diverged by one and two mutations from the most common haplotype (Leonhardt et al. 2019).

Gene flow is not always strongly limited between subterranean populations. In a cave cricket from the Rhaphidophoridae family, *Dolichopoda schiavazzii* Capra, 1934, twelve populations from caves and man-made subterranean environments in the Apennine Peninsula, as well as on the islands Elba and Pianosa, were studied (Allegrucci et al. 1997). Gene flow was not observed between the island and continental populations; however, between the two populations that occur in the caves on the Monte Argentario promontory, substantial gene flow was noticed. It is believed that genetic variation was influenced more by the degree of isolation and dependence on climatic factors than geographical distance (Allegrucci et al. 1997). A similar conclusion was observed in fish populations from the Cyprinidae family *Garrabarreimiae* Fowler & Steinitz, 1956 inhabiting cave water and populations inhabiting surface water in Oman (Kruckenhauser et al. 2011). A substantial genetic difference between the populations was explained by a founder effect in the cave. However, some gene flow was observed, which was explained by the occasional contact between the subterranean and surface populations. On the other hand, the results obtained by Boyd et al. on cave beetles (2020) suggest that isolation by distance influences genetic structure.

The obtained results support a widely accepted theory that underground living species, as well as introduced ones, have a lower genetic variation in comparison to ancestral populations. For a better understanding of changes that have recently occurred in the introduced *Speonomus normandi hydrophilus*, it seems necessary to use microsatellite markers or NGS technology (Fumagalli et al. 2013, 2014) in further studies. These techniques and approaches allow tracking recent changes in the genome resulting, for example, from changes in the population size (Garza and Williamson 2008), colonisation of a new area (Estoup and Clegg 2003; Zepeda-Paulo 2010), or gene flow (Bradic et al. 2012).

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